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Inhalation Toxicologic Procedures for Exposure of Small Laboratory Animals to Highly Toxic Materials:
Part A: Exposures to Gasses with Notes on the Toxicity of PFIB

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INHALATION TOXICOLOGIC PROCEDURES FOR EXPOSURE OF SMALL LABORATORY ANIMALS TO HIGHLY TOXIC MATERIALS: PART A: EXPOSURES TO GASSES WITH NOTES ON THE TOXICITY OF PFIB

by

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DRES ANIMAL CARE STATEMENT

In conducting the research described in this report, the investigators adhered to the "Guide to the Care and Use of Experimental Animals" published by the Canadian Council on Animal Care.

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ABSTRACT

The estimation of military hazard from toxic materials usually requires an estimate of the human toxicity. With expensive, rare and highly toxic substances, only small amounts of substance may be available for testing or safety considerations may restrict the amounts of material that may be used at one time. A functional system has been developed for the exposure of animals to highly toxic gas or aerosol test materials. Mice may be exposed either nose only or whole body. Rats, guinea pigs and small numbers of larger animals may be exposed nose only. Provision is made to continuously monitor the exposure concentrations of either gasses or aerosols in the test atmospheres. Respiration of the subjects may be continuously monitored during the exposures but because of the nose only configuration, the restraint of the animals precludes observations of behaviour during the exposures.

This report covers the system as configured for recirculating exposures to air dissolved chemicals (gasses). Other configurations, covered in companion reports, permit dynamic exposure to both aerosols and gasses. Operation of the recirculating system is described using toxicity estimations to perfluoroisobutylene to CD-1 mice as the trial example. The LCt_{50} value obtained was 960 mg.min/m^3 for a 10 min exposure, a value that agrees with data generated in other laboratories.

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EXECUTIVE SUMMARY

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BACKGROUND

Estimation of the toxicity of inhaled vapours and aerosols and an estimate of the toxicity to man are a basic part of military hazard assessment. For military purposes, the estimates required are usually for highly toxic materials and, complicating the issue, materials that are in very short supply.

A system was required that would provide inhalation toxicology values for very toxic materials, using small volume chambers both for safety and thrift reasons, that would be flexible enough to handle both gasses and aerosols and provide continuous recordings of respiration and chamber concentration. What has been developed is an exposure system that provides for these needs, is readily and relatively inexpensively made, can be quickly decontaminated, will allow use of a variety of research animal species and by various rearrangements, will operate with gasses and aerosols in both recirculating and dynamic (flow through) exposure modes.

CAPABILITIES OF THE SYSTEM

The total system may be configured for either whole body exposures to mice or nose only exposures to mice, rats, guinea pigs and ferrets. The system may operate in recirculating or dynamic modes according to the needs of the particular trials. Because many of the components are plastic, some modifications are necessary for trials with materials that are not compatible with these components. In this case, steel and glass modules are substituted for the plastics.

The modular system configured for recirculating gas exposures, will permit the estimation of toxicity (LCt_{50}) in a variety of laboratory species, while using minimal amounts of test material (*eg.* 20 mL gas or 100 μL liquid that valorizes). Mice, rats, guinea pigs and ferrets are readily accommodated for nose only exposures and mice may be exposed whole body. A continuous record of the concentrations in the test atmosphere is created. The respiration of the animals can be followed during nose only exposures. Exposures of 5 - 15 min are readily performed and longer exposures are possible with due care for the welfare of the test subjects.

The system has been in use for several years at Defence Research Establishment Suffield for a wide variety of studies but has never been described in detail in a general forum. This paper describes the system as configured for recirculating gas exposures including operating capabilities and parameters using exposures to perfluoroisobutylene (PFIB) as a working example. The LCt_{50} for PFIB is estimated to be 960 mg.min/m^3 for CD-1 mice exposed for 10 min.

INTRODUCTION

Inhalation toxicology is a basic part of military chemical warfare hazard assessments. Assessment of the military hazard posed by inhaled vapours requires estimation of the toxicity of chemicals inhaled by man. Estimations of the human dose response are usually made by extrapolation from data describing the toxicity observed in a number of laboratory animal species [1, 2, 3, 4, 5]. Because of the expense, in both specie and manpower, associated with such studies, chemicals are often first screened for toxicity in mice and further testing is done only for those substances that prove toxic or are of commercial interest.

In recent years, the use of experimental animals has come under more and more scrutiny and the acceptance of older methods has been questioned because of the numbers of animals used. Concomitant with this change, the requirement has arisen to provide toxicologic data from small to minute amounts of test material, either because of cost, extremely limited availability of material or extreme toxicity. The result has been a search for safer and more efficient methods for toxicologic testing and a re-evaluation of static and recirculating methods of exposure as means to provide the needed technology. From the data presented in Table I, the potential of these alternate methods is clear.

Older, and now almost *passé*, techniques exposed large (>250) numbers of animals "whole body" to air dissolved or aerosolized materials. Newer techniques expose fewer (50) animals "nose only" to directed streams of test materials. Both of these methods, as commonly employed, are usually referred to as "dynamic" because a constant flow of "fresh" atmosphere is presented to the animals to minimize problems associated with O_2 depletion, build up of CO_2, NH_3 and other metabolites and changes in the test atmosphere caused by metabolism of the test chemical. Such systems, even when the volumes are reduced as much as possible, utilize relatively large amounts of test chemical (kg). When only small amounts of test chemical are available, use of a recirculating system or

Table I

Material required for one exposure at 1.0 mg/m^3 for 10 min

System	μg
100 L dynamic chamber (1000 L/min flow)	10,000
6 L dynamic chamber (60 L/min flow)	500
12 L recirculating system	12
5 L static system	5

even a "dead-air" closed chamber may be the only practical method of obtaining the needed toxicity data.

In addition to the incentives listed above, there was a need for inhalation exposure systems for use with highly toxic materials that could be readily cleaned and decontaminated or, in the worst case, disposed without undue cost or program disruption. These goals have been achieved with the system described. The same modular system can be configured for exposures either to gasses or aerosols either whole body to mice or nose only to mice, rats, guinea pigs and ferrets. The parts are either readily obtainable on the open market or easily constructed from plexiglass by any machine or fabricating shop. The test atmosphere can be continuously monitored and, as an added bonus, respiration monitors can be used with the system.

This report describes the system as used for recirculating exposures to air dissolved chemicals (gasses). The companion reports [6, 7] describe the system as configured for dynamic aerosol and gas exposures.

MATERIALS AND METHODS

Chemicals

FREON-12 was purchased from Matheson Gas Products Canada, Edmonton, Alta. Perfluoroisobutylene (PFIB; CAS#382-21-8; MW 200) was purchased from Flura Corp., Newport, TN. Phosphate buffered isotonic saline (Dulbecco A) was prepared as sterile solution from a prepared commercial powder mixture (Oxoid Ltd., England).

Animals

The male mice, rats and guinea pigs used in this study were outbred Crl:CD®1(ICR)BR, Crl:CD®(SD)BR and Crl:(HA)BR strains, respectively. These animals were purchased from Charles River Canada, St. Constant, Que and were acclimatized for at least 7 days in the Vivarium at Defence Research Establishment Suffield (DRES) before use. The ferrets (Black faced/fitch ferrets; *Mustela putorius*) were purchased from Marshall Farms, North Rose, New York.

Air supply

Medical grade compressed air (Zero gas) was purchased either from Praxair Products Inc., Mississauga, Ont. or from Canadian Liquid Air, Calgary, Alberta.

Particle and chemical free air was supplied by a breathing air system consisting of a SIHI model KHPE 35504 water sealed compressor (SIHI-Halberg International Co., Ludwigshafen/Rhein, West Germany) fitted with HEPA filters on the air intake, a water separator and air dryer immediately following the compressor and then oil and particle filters in the compressed air line. The result was dry, particle and chemical free air delivered at 60 lb/in^2 to the inhalation exposure system.

Respiration monitoring

As each animal holder on the nose only exposure chamber is, for practical purposes, a closed volume, the nose only animal holders could be connected to a respiration rate monitor (DATAMAX-8, Columbus Instruments Int. Corp., Columbus, Ohio) for continuous monitoring of respiration during the exposures. With the equipment available, 8 of the 10 possible animal holders could be monitored at any one time. Data gathered from the DATAMAX was transferred to spreadsheet files on FRAMEWORK® (Selections & Functions, Inc., Scituate, MA 02066) for data reduction, manipulation and statistical analysis[1].

Calculations and Statistics

Raw particle characterization data were assembled into tables of sample number, time and particle number/mass using programs written for FRAMEWORK®. Various summary tables were then prepared according to the needs of the experimental work.

Calculation of the LCt_{16}, LCt_{50} and LCt_{84} values[2] were done using USEPA developed [8] and commercial programs [9, 10] and a newer process developed in this laboratory [11]. All processes utilize PROBIT analysis but the Yee approach [11] employs a newer, more powerful non-linear optimization procedure to fit the PROBIT relationship to the experimental data and so can provide analyses of data which cannot be handled by the earlier Bliss [8] or Litchfield & Wilcoxon [9] methods.

Mean body weights and lung wet weights and proportional weights were compared, first using analysis of variance (F-test) and then, to ascertain which groups were different, using

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[1] FRAMEWORK IV, originally developed by Ashton Tate, was not supported when Borland purchased that company. A new version, FRAMEWORK VI, which runs under Windows 95 is now available from Selections & Functions, Inc., Box 505, Scituate, MA 02066

[2] LCt_{xx} is the Concentration - time product that produces xx percent Lethality under the defined conditions. Concentration refers to the inhaled air and is usually in mg/m^3 for military purposes. For military purposes, time is usually given in min. Hence the usual units of the LCt_{xx} are $mg.min/m^3$.

serial two-tailed Student's *t* tests [12], were compared using analysis of variance followed by one-tailed Student's *t* tests [12].

DESCRIPTION OF THE INHALATION EXPOSURE SYSTEM

General

The inhalation exposure system is modular and may be rearranged to suit the needs of the moment. There are two exposure chambers, one for whole body exposures to mice and one versions for nose only exposures to mice, rats, guinea pigs and ferrets. The whole body chamber was intended for mice but hamsters could also be accommodated. The nose only chamber was a universal chamber with a separate animal holder which can be adapted for use with mice rats and guinea pigs. Hamsters could be easily accommodated but have not been used to date. A similar, longer animal holder was used for the exposures to ferrets.

Each chamber has sampling ports, made of Swagelok® union or bulkhead fittings with septa inside the caps, mounted on the upper faces of the ends for extracting grab samples of the exposure atmospheres. In the usual recirculating operation (*vide infra*), a MIRAN Analyzer is placed in the loop before the chamber to monitor the chemical concentrations in the exposure atmosphere.

Exposure chambers

Two chambers were developed of ≈6 L capacity. One chamber was intended for whole body exposures to mice (Fig. 1). The second chamber developed (Fig. 2) was a "Universal" chamber intended for nose only exposures with all common laboratory species.

Both 6L chambers were Plexiglas boxes with squared "conical" ends. The test atmosphere was introduced at one end through an 0.5 inch Swagelok® O-Seal Threaded Male Connector and exhausted at the other through a Swagelok® union fitting.

Whole body mouse chamber

The whole body chamber (Fig. 1a) has an air-tight lid that opens the entire length of the body of the chamber. The animals are held in a metal mesh, 10 compartment "condo" (Fig. 1b) which fits closely into the body of the chamber.

Nose only chamber

On the nose only chamber (Fig. 2), the lower edge of each chamber side has a guide into which the front plate of the animal holder is seated. The upper edge of the chamber has "butterfly" nuts that are turned to hold the holder in place. A face seal of dental dam closes the animal ports. With this chamber, up to 10 mice or rats, 8 guinea pigs or two ferrets may be exposed at one time in a recirculating mode for up to 20 min (mice could be exposed for 30 min). The ferret holders were usually mounted nearest to the exhaust from the chamber (Fig. 3).

Animal holder for nose only exposures

The central part of the current nose only exposure system is the animal holder, made of Plexiglas, which easily clips onto the exposure chambers and is readily adapted to hold mice, rats and guinea pigs. The animal holder (Fig. 4) was constructed and machined from plexiglass tubing and sheet. The body of the holder for small laboratory animals is a 6.1 cm inside diameter 17 cm long tube. The inside diameter was chosen specifically to provide a close but not tight fit for a modified 250 mL polypropylene conical centrifuge tubes used as a disposable sleeve (*vide infra*). A flat plate is attached at one end as the junction with the exposure chamber. The plate has a 4.9 cm hole, centred on the attached tube, which receives the animal nose and guides and positions it in the exposure chamber. At the opposite end there is a threaded closure with an o-ring seal and, centred, a plunger attached via a drilled out 0.25 inch Swagelok® union. The plunger has a circular tip sized to match the animal being exposed with a flattened area to allow the tail of the animal to pass the plunger.

For rats and guinea pigs, a sleeve, cut from a 250 mL polypropylene conical centrifuge tube, is used to restrain and position the heads and noses. For mice, a 50 mL conical centrifuge tube sleeve is used with spacer washers to keep the tube centred in the holder. The conical tips of the sleeves are cut off to make a hole of appropriate size to fit the nose of the species being exposed. When the animals are placed in the holders with the sleeves in place, the conical tip fits into the side ports of the exposure chamber, positioning and holding the nose in the exposure atmosphere and preventing the animals from turning or withdrawing their heads.

Because the holder is essentially an isolated air space, a respiration monitor can be attached to trace the respiration rates and volumes of the exposed animals. A Swagelok® union fitting is mounted on the centre top of each holder to facilitate connection to a respirometer.

For ferrets, a similar animal holder was assembled with the tubing 44 cm long. These holders could be used with ferrets < 1.1 kg in body weight (Fig. 3).

Exhaust air

When the system is used with chemical atmospheres, the purge/exhaust air from the chamber system is passed through a Canadian C2 Mask Canister (NATO Stock No. 4240 21 871 7842) adapted to the system by a Teflon adapter threaded for the mask canister and fitted with a Swagelok® union fitting to mount on stainless steel tubing connected to the chamber end port.

The Recirculating Exposure System

For recirculating operation, the system components are arranged (Fig. 5) in a loop configuration around a flow control valve (4-way high flow ball valve; Whitey Valve No. SS 45YF8) used to select recirculation or purge air flow.

When the Control Valve is closed the system is in the recirculation mode and the contained air is pumped continuously through the injection port, into the MIRAN, to the animal chamber, to the Control Valve and returns to the pump. The recirculation pump is a Metal Bellows diaphragm pump with a 15 L/min output. An injection port, a Swagelok T-fitting with a septum on one leg of the "T", is mounted on the output port of the Metal Bellows pump.

When the Control Valve is open, the system is in the "purge" mode and fresh air is drawn continuously from the laboratory and exhausted through a military mask filter.

OPERATING THE SYSTEM

Introduction of test substances

Gasses: To introduce gas samples of >0.5 mL volume into the system, an amount of gas was first transferred from the storage cylinder into an SKD Tedlar® gas sampling bag (Supplied by Safety Instruments Ltd., Edmonton, Alberta), aliquots of the neat gas for injection were drawn from the gas filled Tedlar® bag using Hamilton Gastight Syringes (Hamilton Company, Reno, Nevada) and injected into the recirculating system. For <500 μL volumes, a one L Tedlar® bag was used with gas samples drawn into disposable laboratory syringes with sealing rubber plungers. All the above syringes were fitted with Gastight Syringe Valves (Hamilton Company, Reno, Nevada) and 3 inch 26 gauge spinal needles. If the sample to be introduced was <25 μL, a gas dilution was usually employed. A known volume of air or N_2 (250 -500 mL) was measured into an empty, one L Tedlar® bag using a large volume syringe (SGE Inc., Austin, TX) fitted with a Gastight syringe valve. Then, an appropriate amount of gas was added.

An aliquot of the diluted gas was then injected into the inhalation system.

Liquids: When the volume to be introduced was > 1 μL, volatile liquid materials were injected directly into the recirculating air stream using Hamilton Microliter syringes (Hamilton Company, Reno, Nevada). When the volume of liquid required was <1 μL, a volume of air or N_2 (250-500 mL) was measured, using a SGE large volume syringe, into a 1 L Tedlar® bag and the appropriate amount of liquid was injected into the bag. Then the sample was treated as a gas sample.

Determining concentrations of test substances

The concentration of air dissolved material in the recirculating exposure atmosphere was continuously measured using MIRAN Analyzer (MIRAN 1A CVF General Purpose Gas Analyzer (Foxboro Analytical, South Norwalk, CT) placed in the air stream before the exposure chamber. Output from the Analyzer was recorded on either a Hewlett-Packard Chart Recorder, Model 7132A (Hewlett-Packard, Palo Alto, Calif.) or a Rikadenki R-56 Multi-pen Recorder (Rikadenki Kogyo Co. Ltd., Japan). The MIRAN Analyzer was operated at the minimum sensitivity/path length that would provide 75% full scale deflection on the recorder with the highest concentration of material in the exposure atmospheres.

With each unknown trial substance, a wavelength spectrum was obtained (Fig. 6) on the MIRAN Analyzer to determine a suitable wavelength for operation. The maximum expected concentration of the test substance was then introduced, the wavelength of maximum absorbency was checked and the path-length and recorder range settings were determined. The Analyzer was then calibrated by sequentially injecting increasing aliquots of the test substance (Fig. 7) to determine the instrument response *vs* vapour concentration. For each peak, the area of one chart division (usually in cm^2) at the equilibrium displacement was measured as the value for that amount of chemical in the system. The system was purged between each injection. When possible, the response range was kept between 0.0 and 0.3 absorbency units so that the instrument response remained sensitive and reasonably linear. During the exposure, the area under the trace for each chart division was calculated and, using the calibration curve, an exposure for that period was calculated. These values were summed to provide an estimate of the total exposure. This is essentially an integration technique. The only caveat is that the recorder ranges and the chart speed during calibration and during exposure must be the same. Generally, the loss of exposure during the equilibration of the system (t_{99}) was compensated by the increase in exposure during the purge. As the error possible appeared less than 5 %, no correction was

made to the nominal exposure time.

When needed, grab samples for external analysis were drawn through septum stoppered ports from the exposure atmosphere using Hamilton Gastight syringes.

Operating procedure

The operating procedure is essentially the same for all systems described.

At the start of each working day, the configuration of the system is checked against the experimental protocol, the values of all air flows are checked and all joints are checked for leaks.

The system is calibrated by the appropriate procedure and a blank or control exposure (no animals) is performed. If warranted, dead animals were also exposed to determine hair/body uptakes.

For each animal exposure, the animals, pre-weighed, grouped and placed in caging according to the experimental protocol of the day, were quickly loaded either into the holders for nose only exposures and mounted on the sides of the exposure chamber or placed in the individual compartments of the whole body exposure "condo". An adequate supply of clean air must be flowing through the system. When respiration monitoring is in use, the monitors were connected as soon as all the animals were in place on the exposure chamber.

At time zero, the clocks and instruments (recorders, respiration monitors) were started and the control valve was turned to start recirculating mode. The system is run for two min to allow final checks and to record baseline data on all instruments. At time 2 min, the test substance is introduced and the development of the test atmosphere is carefully monitored thereafter. At the end of the prescribed exposure period, the control valve is turned to purge the system. When the instruments indicate that the atmosphere in the exposure chamber is essentially cleared of trial substance (usually 8 min after the start of purge), the animals were removed and evaluation began.

In all cases, the timing of events was started (time zero) when the trial substance was introduced. Behaviour of the animals exposed "whole body" may be monitored continuously during exposure. Unfortunately, no behavioural data can be collected from animals held "nose only" because of the restraint. Thus, with nose only exposures, the first observations on the test animals will be made about 8 min after the end of the exposure when the animals are removed from the holders. Respiration monitoring, in contrast, can continue throughout exposure and purge.

The animals are observed continuously for the remainder of the working day, weighed and examined each morning for the rest of that work week and twice a week thereafter until at

least the 21st day. Casualties were examined post-mortem and tissues were examined histologically as stained paraffin sections.

The usual exposure times for this system operating at DRES were 10 min. The exposure times may vary within certain limits. However, exposure times <5 min have a significant proportion of the exposure in the start-up (t_{95}) and purge periods and, therefore, the lower time limit is set by the ability to obtain accurate exposure rates. For longer exposure times, the t_{95} becomes less of a factor as the time increases and other factors become limiting. For whole body, recirculating operation, the limiting factors for longer exposures are the supply of O_2 and the buildup of CO_2. These factors are dependent upon the recirculating volume. With mice breathing at 25 mL/min, there appears to be no problem with 30 min exposures with 10 mice. With 5 rats or guinea pigs, the 30 min time appears to be the extreme limit. For nose only exposures, an additional factor is the heat build-up for the animals confined in the holders.

Calculation of exposure

In several control experiments with FREON atmospheres it was determined that the difference between the exposure calculated assuming the exposure profile to be a square wave and the exposure calculated by integration of the recorder tracings was less than 5%. Thus, the extra exposure obtained as the concentration falls during the purge cycle and the loss of exposure during the starting t_{95} period essentially cancelled each other.

A nominal exposure is quickly obtained by calculating the dilution of the chemical introduced into the volume of recirculating air. The actual exposure is best calculated from the recording of the MIRAN Analyzer. When there is no significant loss of chemical from the atmosphere during the exposure (Fig. 8), the actual exposure will be close to the nominal value. Some small error may result from errors in the injection and variance caused by t_{95} up and purge effects. When there is uptake of trial chemical either by the system, by the animals or by decomposition, the difference in the exposure values in the presence and absence of animals may be used to obtain a value for the uptake of trial material.

LIMITATIONS OF THE GAS EXPOSURE SYSTEM

The major limitations of this system are concerned with the stability of the trial materials and the possible interaction of the trial materials with the plastic parts of the chambers. The trial gas in the exposure atmosphere must not be consumed at a rate that will negate the animal exposures and the interaction between trial substance and structural plastics must be minimal.

As most per acute toxicities of military importance are involved with highly lethal/effective materials, the amounts of material involved are relatively low and many factors can combine to reduce the substances in the exposure atmosphere. Not the least of these is the uptake of the chemical by the living body - not necessarily involved with toxicity. We have encountered one chemical that had to be assessed dynamically because the uptake was so fast that a stable atmosphere could not be obtained in the recirculating system. The uptake of chemical by the animals may also be passive *eg.* uptake by adsorption on the hair. Some materials (nerve agents) are absorbed by plexiglas and no useful data can be garnered from the exposure chambers described. Nevertheless, the exposure systems described have stood up to most of the trials required since their construction. The ability to obtain toxicity values from very small amounts of material far outweighs the few drawbacks that have been identified to date.

RESULTS AND DISCUSSION

Relationship between exposure and dosage

With air dissolved chemicals, the exposure and dose are directly related by the uptake of the chemical from the air assuming that the animal does not alter it's respiration mechanics. The relative solubilities of the chemical in air and lung fluids and the transport of the chemical across cell membranes are the governing factors. In calculating an LCt_{50}, the usual assumption is that all the chemical is absorbed. LD_{50} values calculated from LCt_{50}'s are therefore numerically higher than LD_{50}'s obtained by injection. When the proportional uptake of chemical can be determined, the LD_{50} obtained by inhalation will approach those obtained by injection methods.

Exposure profiles

The chambers described have been used for exposures to air dissolved chemicals. Exposure atmospheres between 100 and 100,000 mg.min/m^3 are easily obtained with this system. The overall utilization of chemical for toxicity testing is, of course, significantly reduced when the recirculating mode is used.

The system is usually operated with a recirculating flow rate of 15 L/min. The following data are examples from recirculating system experiments.

The t_{95} was consistently between 0.75 and 0.9 min when FREON-12 or PFIB were introduced in the absence of animals (Fig. 8). The concentration of FREON-12 was constant once equilibration was achieved and could be used as a performance/leak check on the system.

Similarly, the concentration of PFIB was constant after equilibrium had been achieved. With mice on the chamber, the concentration of FREON-12 was not affected. The concentration of PFIB, however, decreased slowly over the 10 min exposure time of the test run. The recorder tracing could be readily extrapolated to time zero to estimate the expected concentration from that injection. During purge, the chemicals were cleared to 1% of the equilibration value within 4 min.

The loss of chemical during the exposure time could be measured easily form the traces. The theoretical equilibrium value was obtained by extrapolation of the trace to time zero and then the measured concentration at any time during the exposure was subtracted from the time zero estimate to obtain the amount of chemical lost. With reactive materials, it may be necessary to estimate the amount of chemical taken up by the hair and external parts of the animals by running an exposure(s) with dead animals in the system.

Rebreathing was not considered to be of concern with the large volume system providing the uptake of chemical was less than 20% of the initial concentration. With one chemical that has been tested, the uptake was close to 40% from the recirculating system. A quick check of the LCt_{50} using a dynamic system produced a value only 10% higher than that produced in the recirculation system [13]. Since Haber's rule[3] was not followed in the exposure range employed, the values were considered equivalent as that level of discrepancy could easily be generated in the integration of the exposure profile. When exposing mice, the sum of the minute volumes of the 10 animals (≈250 mL [4, 5, 14, 15]) is a small proportion of the 12.6 L volume of the recirculating system and uptake rates must be extreme to remove 20% of the chemical (but it does occur [13] *eg.* with HCN). When rats or guinea pigs are to be exposed, the larger minute volumes of these animals (250 and 460 mL, respectively) means both proportionably larger uptake values and that the proportion of air breathed/min is becoming critical. To compensate, the numbers of animals per exposure group was usually reduced to 4 - 6. Similarly, with ferrets (minute volume 0.6 L/min for a 1 kg animal [16]), a maximum of two animals were exposed at one time.

Example of toxicity studies; PFIB

The per acute toxicity of PFIB to CD-1 mice was estimated using the large chamber recirculating system. Both male and female mice were exposed. However, there was no apparent

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[3] Haber's rule states that the LCt_{50} is constant as the exposure time changes. This is necessary for accurate calculations of exposure by integration of the exposure profile In most cases, the deviation of $LCt_{50} = C$ is small within the range of exposures encountered in the systems discussed.

difference in the toxicity so the groups were combined for the PROBIT calculations. The plot of mortality vs exposure concentration was very steep (Fig. II, III). Toxicity (LCt_{50}) estimates were made using the Bliss [8] (USEPA version), Litchfield/Wilcoxon [9] and Yee [11] methods (Table II, III).The estimated LCt_{50} value was 96 mg/m^3 for a 10 min exposure or 960 mg.min/m^3. The PROBIT slopes were more variable between the three calculations but all were very steep. The LCt_{50} value is in essential agreement with values from other laboratories [17, 18, 19]. The differences between the calculated values obtained with the three methods serve to emphasize, again, the difficulties inherent in providing toxicity values removed from the LCt_{50}.

The amount of chemical used to obtain these results, including calibration of the systems, was ≈20 mL of pure gas at STP. During a typical exposure at the LCt_{50}, 10 mice took up about 10% of the PFIB from the recirculating atmosphere.

The times to death were usually between 24 and 48 hr (Table II, III). The last casualty died overnight on day 3 and so was scored as dead on day 4. Lung edema was the cause of death in all cases. No other pathology was recorded.

All animals exposed to PFIB lost body weight in the first 2 days post exposure (Table II, III). Recovery from the exposure insult was slow. Animals exposed to lower levels regained the weight lost and then matched the control gains by the end of the 23 day observation period. Survivors exposed to near LCt_{50} levels of PFIB did not regain weight lost albeit that the animals did not appear to be unhealthy.

SUMMARY

The equipment and procedures are described for estimation of inhalation toxicity in laboratory animals (mice, rats, guinea pigs and ferrets) while using mg quantities of volatile chemical. The toxicology of PFIB is presented as a working example of the techniques.

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Table II

Toxicity of PFIB to CD-1 mice

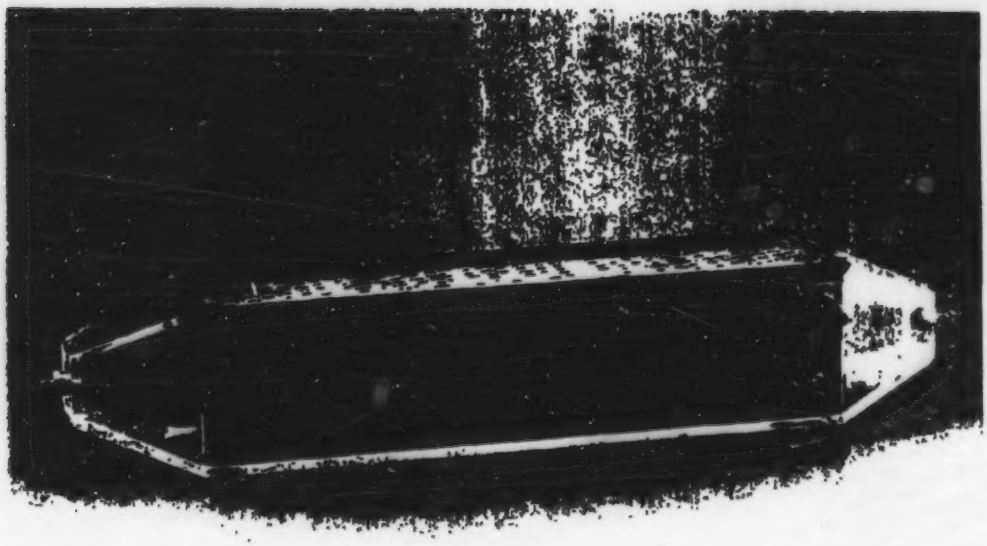
Exposure (mg/m^3)	mortality	Number deaths by day				Body weight	Change in Body weight by day post exposure							
		1	2	3	4		1	2	3	4	7	13	22	29
68	0/10	.	.	.	.	23 9	5.5	5.0 ±1.4	8.3 ±5.6	3.4 ±5.5	14 ±8.7	19 ±12	.	.
75	0/10	.	.	.	.	25.6 ±1.7	3.6 ±3.0	3.8 ±4.4	6.2 ±3.6	8.0 ±4.7	8.3 ±4.0	12 ±6.9	16 ±9.6	18 ±8.1
85	0/10	.	.	.	.	26.4 ±1.9	1.5 ±4.3	-2.9 ±5.0	0.8 ±5.8	2.3 ±4.8	4.8 ±4.4	9.6 ±4.4	13 ±9.4	20 ±8.7
84	2/10	.	2	.	.	24.8 ±2.0	6.2 ±5.3	8.5 ±7.8	3.0 ±6.8	1.8 ±3.8	6.7 ±5.1	11 ±3.8	17 ±5.3	19 ±3.6
96	2/5	5	2	.	.	26.2 ±1.8	-11 ±1.6	-17 ±2.9	-5.2 ±3.0	7.2 ±4.1	20 ±2.7	29 ±4.6	38 ±6.9	45 ±5.6
96	1/5	5	1	.	.	38.1 ±4.7	-7.6 ±2.2	-13 ±3.2	-14 ±4.3	-9.8 ±5.2	-7.9 ±4.6	-6.6 ±6.1	-1.4 ±2.7	-0.7 ±9.2
108	5/5	1	1	2	1	42.1 ±3.2	-13 ±13.6	-10.3 ±3.1	-12					
108	4/5	.	3	1	.	40.0 ±5.0	-7.6 ±1.7	-14	-19	-27	-32	-25	-15	-13
126	9/9	5	4	.	.	36.7 ±4.2	8.4 2.4							
126	8/8	4	4	.	.	38.2 ±2.1	7.0 0.8							
533	10/10	10	.	.	.	24.8 ±1.71								

Table III

PROBIT calculations for PFIB toxicity data

Parameter	Bliss	Litchfield & Wilcoxon	Yee[a]
LCt_{05}	822 (736:869)		822
LCt_{16}	872 (807:914)	857 (854:860)	875
LCt_{50}	963 (919:1019)	964 (896:1037)	963
LCt_{84}	1064 (1008:1181)	1085 (1081:2037)	1060
LCt_{95}	1128 (1056:1299)		1128
Slope	23.96	19.43	23.96
Intercept	-42.53	-33.56	-47.53

[a] The Yee program does not calculate fiducial limits at this time.



A

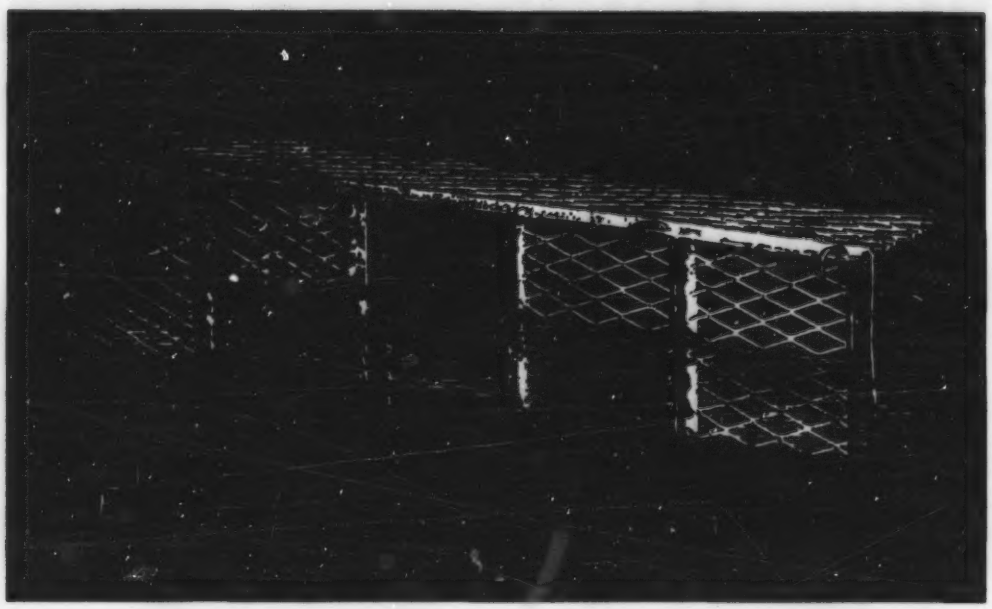


Fig. 1 The whole body mouse chamber and the "condo". In (A), the chamber is shown with the "condo" in place. In (B) the "condo" is shown with two of the side doors open and the wire lock in front. Ten mice were housed in the condo which slips with little clearance into the chamber. The lid is closed and sealed for exposure. Grab samples of the atmosphere may be taken through a septum stopper in one end panel.

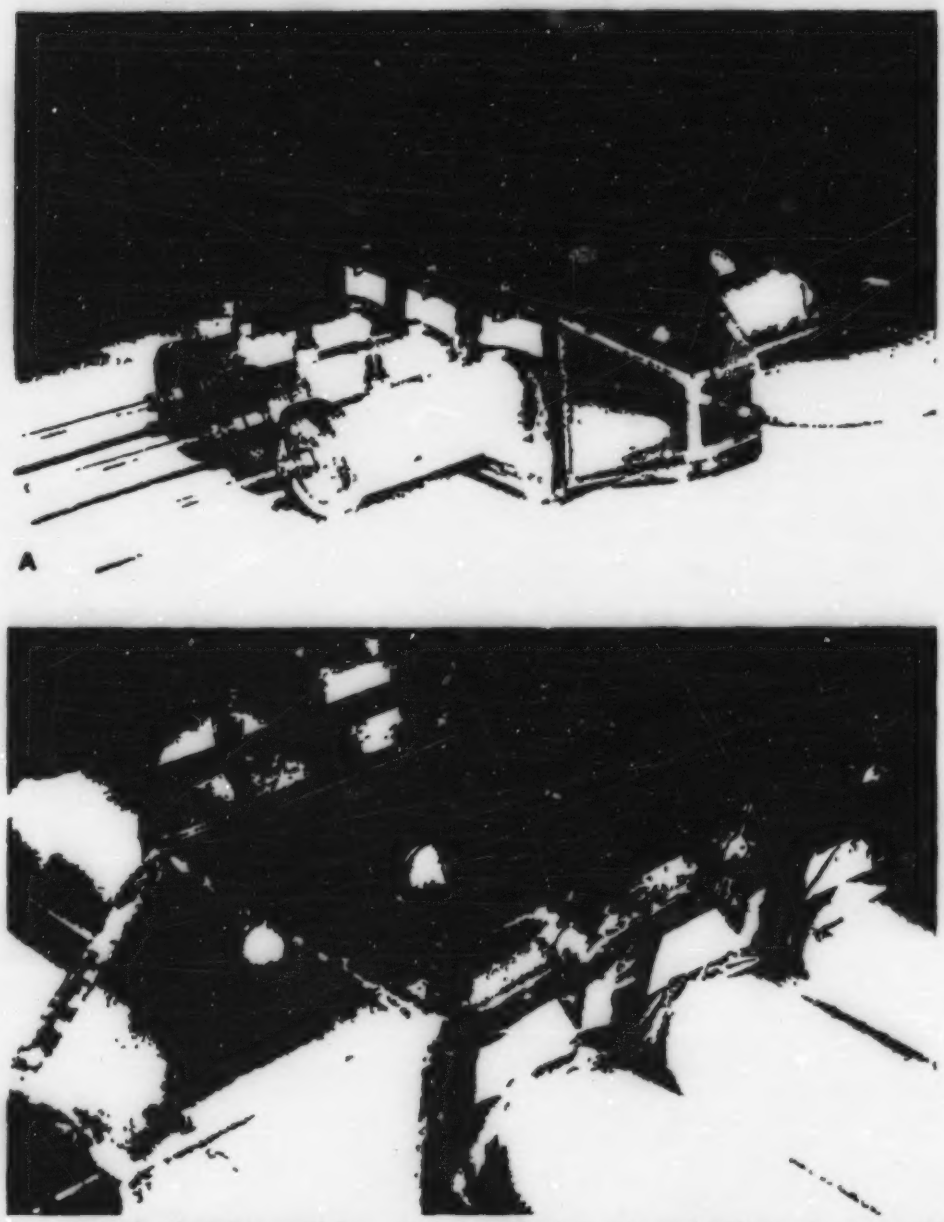


Fig. 2 The nose only chamber used for exposures of mice, rats and guinea pigs to gasses in recirculating mode. In the overall view (A), 6 animal holders containing rats have been mounted. The air flow enters from the rear and exits forward. The holders are held in place by the butterfly fasteners and fit into a matching groove in the bottom of the box side. A later modification installed an airstream diverter in the inlet end to disperse the incoming air. In the close interior view (B), the rat's noses and muzzles can be seen protruding into the exposure chamber.

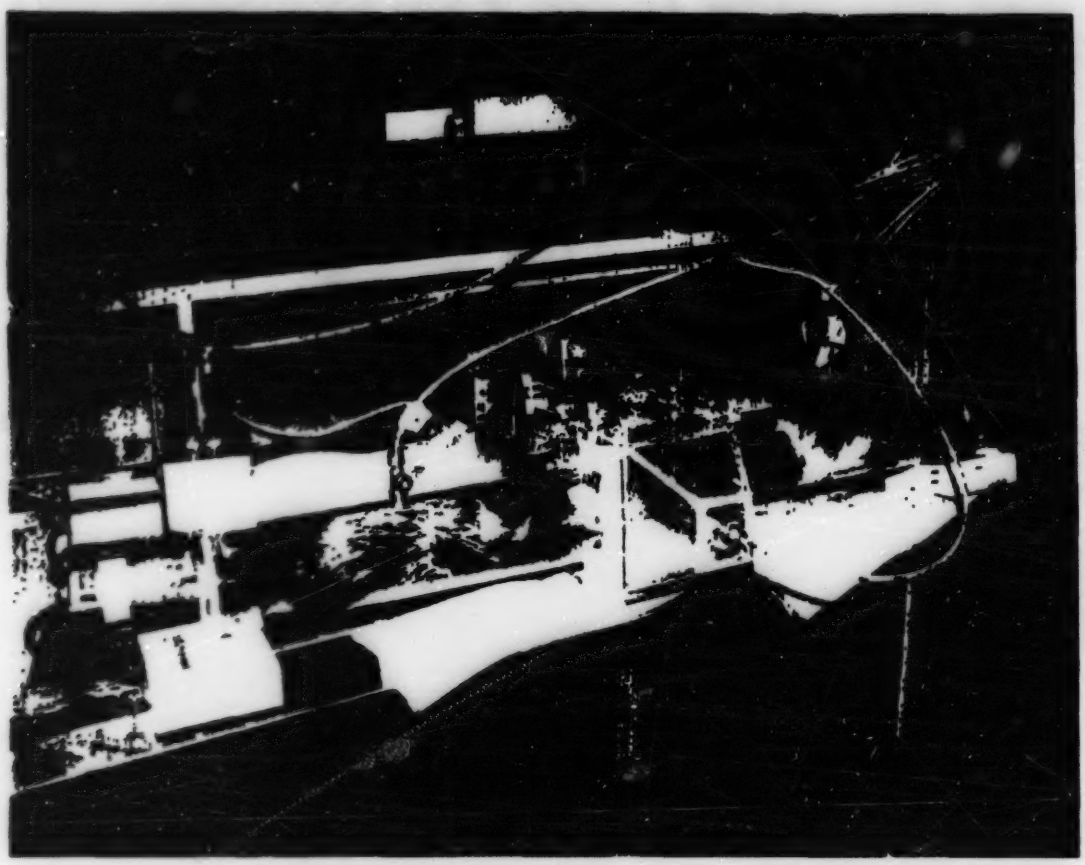


Fig. 3 Ferrets mounted in the inhalation system in the special long holders for this species. The tube diameter is the same as in the shorter small animal holders. Two ferrets are exposed at one time, mounted in the most distal positions from the gas input to the chamber.

Fig. 4 The component parts of the animal holder. Proceeding clockwise from the bottom left are; a guinea pig sleeve, a rat sleeve, a threaded end cap fitted with a mouse plunger tip, the main body of the animal holder, a threaded end cap fitted with a rat/guinea pig plunger tip and a mouse sleeve with it's spacer washers. The main body of the holder has the dental dam on the face plate and the port for connection of the respirometer on the top surface of tube (a Swagelok® fitting closed by a septum (from a gas chromatograph injection port)). The threaded end caps have an O-ring seal that presses against the end of the holder tube.

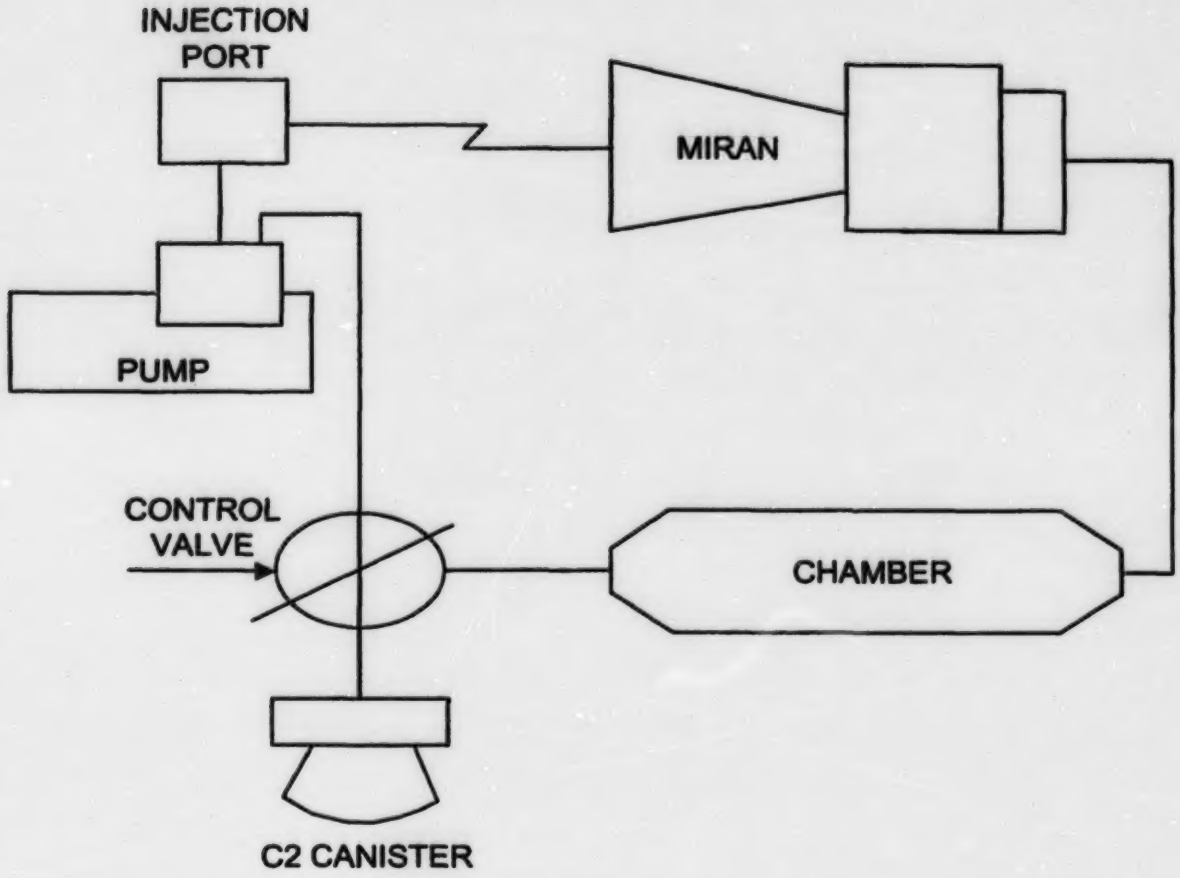


Fig. 5 Arrangement of components for dynamic chemical system

Fig. 6 A typical wavelength spectrum from the MIRAN Analyzer. There are three peaks characteristic of this chemical of which the tall peak at 9.2 μm would be used for analysis.

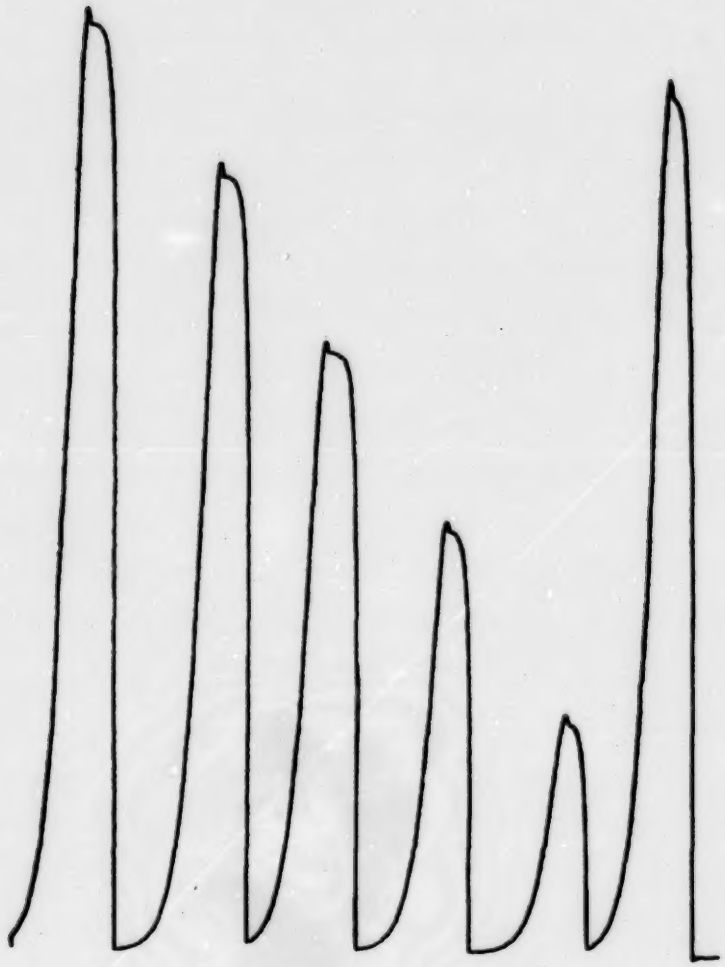


Fig. 7 A calibration trace from the MIRAN Analyzer. The peaks are, in order from right to left, a control resulting from injection of≈4.8 mg, and the peaks resulting from injection of 1, 2, 3, 4 and 5 mg chemical. The peak height in chart units is plotted against either the mg added or the concentration to provide a calibration curve for that chemical under the specified conditions. Alternately, the area of one chart division at the maximum displacement may be used to reduce the effort in integration of the exposure value.

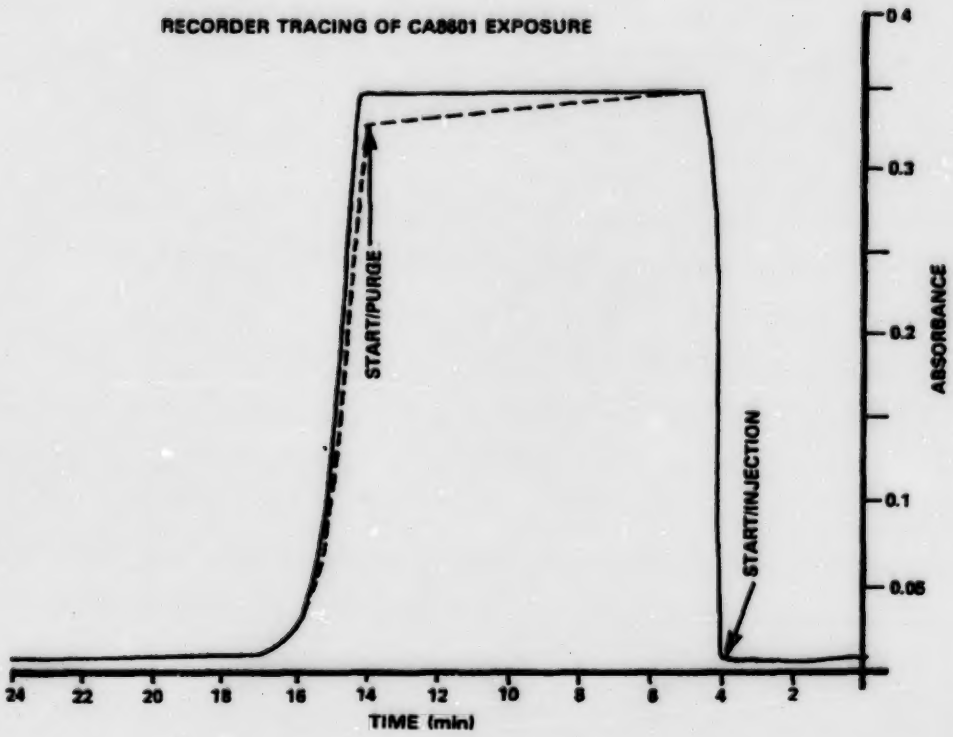


Fig. 8 A recorder tracing from a blank superimposed upon a PFIB exposure experiment. The solid line shows the recirculating chamber system with PFIB and no animals with a stable atmosphere. The dashed line (----) shows the same atmosphere with animals present in the system. There is a small uptake by the animals, the sum of which may be estimated from the difference in the two traces at the start of the purge.

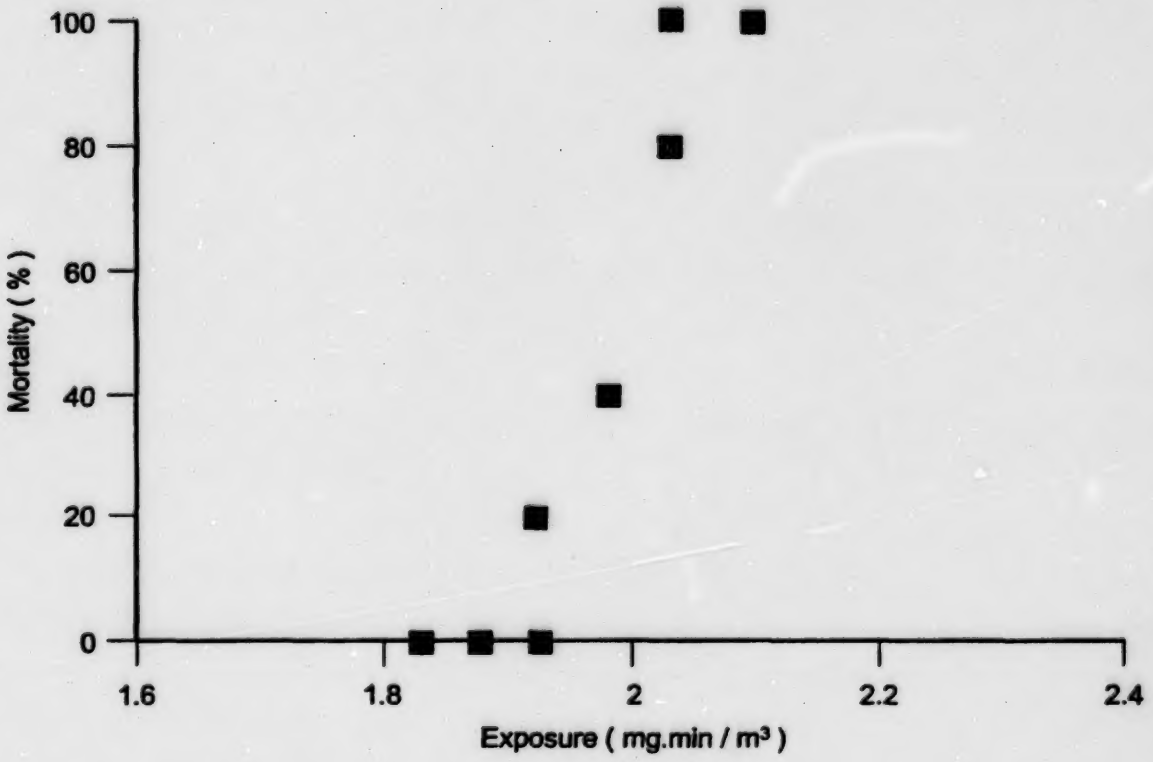


Fig. 9 The mortality resulting from increasing exposures to PFIB. Groups of mice were exposed for 10 min to increasing concentrations of PFIB and then examined daily for 23 days. The last casualty died on the evening of the 3 rd day (scored as dead on day 4).

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The estimation of military hazard from toxic materials usually requires an estimate of the human toxicity. With expensive, rare and highly toxic substances, only small amounts of substance may be available for testing or safety considerations may restrict the amounts of material that may be used at one time. A functional system has been developed for the exposure of animals to highly toxic gas or aerosol test materials. Mice may be exposed either nose only or whole body. Rats, guinea pigs and small numbers of larger animals may be exposed nose only. Provision is made to continuously monitor the exposure concentrations of either gasses or aerosols in the test atmospheres. Respiration of the subjects may be continuously monitored during the exposures but because of the nose only configuration, the restraint of the animals precludes observations of behaviour during the exposures.

This report covers the system as configured for recirculating exposures to air dissolved chemicals (gasses). Other configurations, covered in companion reports, permit dynamic exposure to both aerosols and gasses. Operation of the recirculating system is described using toxicity estimations to perfluoroisobutylene to CD-1 mice as the trial example. The LCt_{50} value obtained was 960 mg.min/m^3 for a 10 min exposure, a value that agrees with data generated in other laboratories.

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